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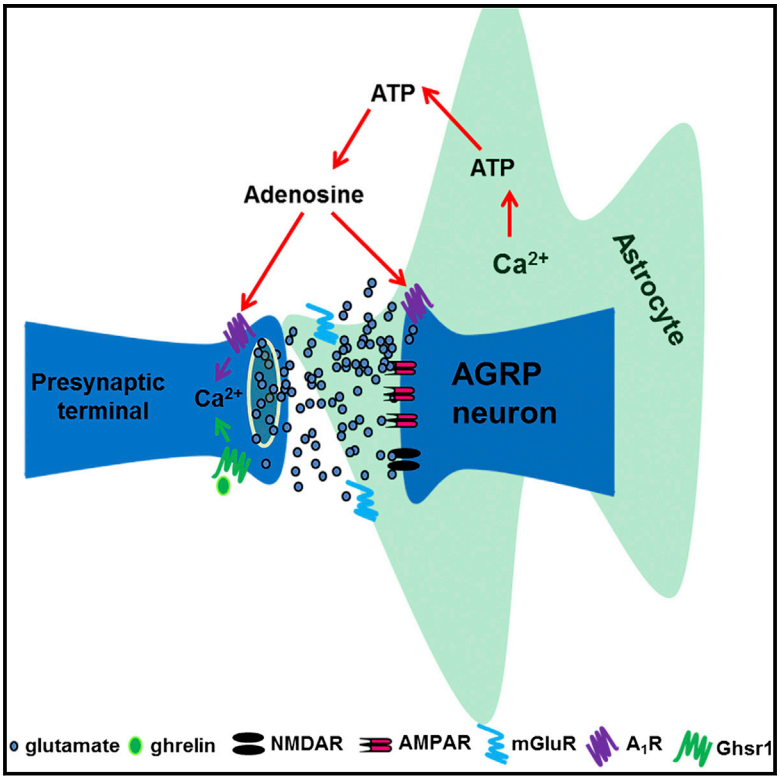
Astrocytes Control Food Intake by Inhibiting AGRP Neuron Activity via Adenosine A_1 Receptors

Graphical Abstract



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In Brief

Yang et al. demonstrate that glial cells bidirectionally regulate feeding behavior in mammals. They find that astrocyte activation depresses ghrelin-evoked hyperphagia, whereas it facilitates leptin-induced anorexia. They also identify a mechanism of astrocytic inhibition of food intake that is adenosine-mediated inactivation of the orexigenic agouti-related peptide (AGRP) neurons.

Highlights

- Astrocytic bidirectional regulation of ghrelin- and leptin-regulated feeding behaviors
- Endogenous adenosine mediates astrocytic inhibition of food intake
- Adenosine A_1 receptors contribute to astrocytic inhibition of food intake
- Astrocytic inactivation of AGRP neurons via adenosine A_1 receptors

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Astrocytes Control Food Intake by Inhibiting AGRP Neuron Activity via Adenosine A_1 Receptors

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SUMMARY

It is well recognized that feeding behavior in mammals is orchestrated by neurons within the medial basal hypothalamus. However, it remains unclear whether food intake is also under the control of glial cells. Here, we combine chemical genetics, cell-type-specific electrophysiology, pharmacology, and feeding assays to show that stimulation of astrocytes within the medial basal hypothalamus reduces both basal- and ghrelin-evoked food intake. This occurs by a mechanism of adenosine-mediated inactivation of the orexigenic agouti-related peptide (AGRP) neurons in the hypothalamic arcuate nucleus (ARC) via adenosine A_1 receptors. Our data suggest that glial cells participate in regulating food intake by modulating extracellular levels of adenosine. These findings reveal the existence of a glial relay circuit that controls feeding behavior, one that might serve as a target for therapeutic intervention in the treatment of appetite disorders.

INTRODUCTION

Neurons that express *Agouti-related protein* are a molecularly defined population localized in the hypothalamic arcuate nucleus (ARC), and they are intermingled with a separate and functionally opposed population expressing pro-opiomelanocortin (POMC). For instance, increasing agouti-related peptide (AGRP) neuron electrical activity is sufficient to rapidly evoke food intake, whereas stimulating POMC neurons exerts negative effects on feeding (Aponte et al., 2011; Atasoy et al., 2012; Kong et al., 2012; Schneeberger et al., 2013). In addition to behaving as interoceptive sensory neurons, whose electrical activities are modulated in response to hormonal signals of metabolic state, both AGRP and POMC neurons receive inhibitory and excitatory synaptic inputs (Liu et al., 2012; Pinto et al., 2004; Yang et al., 2011). In particular, the synaptic inputs that orchestrate the firing rate of AGRP neurons are adaptable to the hunger states (Liu et al., 2012; Yang et al., 2011).

An extensive literature shows that glial cells exert crucial functions in the formation, operation, and adaptation of neuronal circuits. For instance, astrocytes, the most abundant glial cells in the brain, are closely associated with neuronal synapses to scale synaptic strength and modulate neuronal circuits (Clarke and Barres, 2013; Sasaki et al., 2012; Stellwagen and Malenka, 2006; Ullian et al., 2001). In addition, astrocytes are adjacent to the cerebral blood vessels and adjust blood supply (Fields and Stevens-Graham, 2002; Takano et al., 2006; Zonta et al., 2003). Furthermore, emerging evidence demonstrates functional roles of astrocytes in complex behaviors, such as breathing (Gourine et al., 2010) and sleeping (Halassa et al., 2009). Interestingly, it was reported that astrocytes within the medial basal hypothalamus possess Ob-Rb leptin receptors in mice (Hsuchou et al., 2009; Kim et al., 2014; Pan et al., 2011; Thaler et al., 2012). Also, a recent study showed that genetic loss of leptin receptor functions in adult astrocytes diminished the leptin-regulated feeding behavior (Kim et al., 2014). We thus proposed that astrocytes may rapidly regulate food intake by rewiring the appetite control circuits within medial basal hypothalamus in the conditions of energy surfeit or deficit, respectively. To test this, we specifically manipulated astrocytes within medial basal hypothalamus to determine the functional roles of astrocytes in regulating food intake and the action potential firing rate of AGRP neurons.

By combining the chemical-genetic designed receptor exclusively activated by designed drug (DREADD) approach with cell-type-specific electrophysiology, pharmacology, and feeding assays, we found that astrocyte activation depressed ghrelin-evoked hyperphagia, whereas it facilitated leptin-induced anorexia. In contrast, astrocyte inactivation potentiated ghrelin-evoked feeding but blunted leptin-regulated feeding behavior. We further examined astrocytic regulation of food intake and determined the underlying gliotransmitter(s). We found that endogenous adenosine mediated the astrocytic inhibition of food intake and of the firing rate of AGRP neurons by activating adenosine A_1 receptors. Based on these experiments, we proposed that glial cells serve as a checkpoint of feeding by modulating the extracellular levels of adenosine to prevent from energy deficit or surfeit, respectively.

RESULTS

Cell-Type-Specific Astrocyte Activation

To investigate a potential role of the medial basal hypothalamic astrocytes in regulating food intake, a chemical-genetic DREADD approach was used in combination with feeding assays. DREADD uses engineered G protein-coupled receptors (GPCRs) activated by ligands selective for these GPCRs. For instance, the engineered human muscarinic type 3 GPCR (hM3Dq) is not responsive to any endogenous ligand but is instead activated by the pharmacologically inert chemical clozapine-N-oxide (CNO) (Armbruster et al., 2007).

The muscarinic receptor variant hM3Dq fused to a red fluorescent protein mCherry was encoded in the astrocytes within medial basal hypothalamus by using a virus incorporating a glial fibrillary acidic protein (GFAP) promoter. To characterize the cell types expressing the hM3Dq-mCherry protein, we immunostained GFAP in the brain sections of the injected mice and found that the vast majority of the cells expressing hM3Dq-mCherry also expressed GFAP (Figure S1A), as previously shown (Chen et al., 2013; Gourine et al., 2010; Perea et al., 2014). To assess whether the hM3Dq-mCherry protein was expressed in the neurons within medial basal hypothalamus, we stained for the neuron-specific enolase (NSE) marker in brain sections from hM3Dq-mCherry-transduced mice using anti-NSE antibody tagged with a GFP. Although the neurons were surrounded by the hM3Dq-mCherry-transduced cells, few of them expressed hM3Dq-mCherry (Figure S1B).

To test whether hM3Dq-mCherry was expressed in POMC or AGRP neurons, we carried out double immunofluorescence analysis: hM3Dq-mCherry in association with GFP-labeled POMC or AGRP neurons in the hM3Dq-mCherry-transduced POMC-EGFP or NPY-hrGFP transgenic mice in the medial basal hypothalamus (NPY-hrGFP mice allow visualization of AGRP neurons by coexpression of NPY and AGRP in these neurons). The proteins of hM3Dq-mCherry were not expressed in POMC or AGRP neurons, but were expressed in the astrocytes adjacent to neurons (Figures S2A and S2B). Thus, hM3Dq-mCherry was mainly expressed in GFAP-positive cells, which are comprised mostly of astrocytes, although we could not exclude progenitor cells expressing GFAP (Garcia et al., 2004). To date, there is no evidence suggesting that progenitor cells exert rapid modulatory effects on synaptic strength or neuron activity, while there is ample evidence indicating that astrocytes, upon stimulation, rapidly regulate synaptic strength and neuron activity. We assume, therefore, that the transduced astrocytes mediated the rapid regulation of feeding behavior under our experimental conditions.

It has been well demonstrated that the stimulatory DREADD-hM3Dq couples through G_q pathway to depolarize neurons (Alexander et al., 2009; Armbruster et al., 2007) and triggers robust calcium elevations in the astrocytes (Agulhon et al., 2013; Fiacco et al., 2007). DREADD-based in vivo activation of the astrocytes was achieved by intraperitoneal (i.p.) injection of CNO (5 mg kg^{-1} in 200 μl saline), as evidenced by the enhanced astrocytic Fos expression in comparison to that of a vehicle control saline or that of the hM4Di-transduced mice (Figure S3). Together, these results indicated that in vivo administration of CNO did activate the hM3Dq-transduced astrocytes, and thereafter we designated this as astrocyte activation.

Astrocyte Activation Reduced Basal and Ghrelin-Evoked Feeding

To assess the ability of DREADD-based activation of the medial basal hypothalamic astrocytes to regulate feeding behaviors, we evaluated the transduced mice by performing feeding assays. After 2 weeks for hM3Dq expression in astrocytes, the mice first received a vehicle control saline (200 μl) by intraperitoneal injection. Food intake was monitored starting at time 0 and ending at time 9 hr post-injection. The saline injections were repeated on the same mice for 2 successive days. Next, the mice were injected with a test solution containing CNO dissolved in saline at a single dose of 5 mg kg^{-1}. We found that DREADD-based in vivo activation of astrocytes did not show an apparent effect on the feeding during the early light period when mice normally consume little (Figure 1A), whereas a markedly reduced food intake occurred during the early dark period when mice usually consume more (Figure 1B). There were no apparent changes of food intake in the mice that were transduced with the control fluorescent protein mCherry (Figure S4A). In addition, we did not observe changes in the 24-hr food intake between CNO and saline injections (Figure 1B). Strikingly, we noticed a rebound of feeding during the light period approximately 8 hr after CNO injections (Figure 1A). These results indicated that stimulation of astrocytes probably produced a rapid inhibition on the feeding within about 8 hr. Notably, this duration of action for a single injection of CNO is consistent with a previous report (Alexander et al., 2009). To test the possibility that the rebound of feeding was caused by the gradually decreased levels of CNO, a second injection of CNO was given to the same mice at approximately 5 hr after the first injection. We observed that the rebound of feeding disappeared after the second treatment of CNO, as indicated by the decreased food intake compared to that of the mice receiving saline injections or the mice transduced with the control protein mCherry (Figure S4B).

Interestingly, astrocytic inhibition of food intake was associated with the animal's tendency to consume food at the start of dark period. We therefore tested whether astrocyte activation would also affect the orexigenic hormone ghrelin-evoked food intake, since the levels of ghrelin in the circulation are elevated during the dark-period onset (Cummings et al., 2002) or when mice are hungry (Cummings et al., 2002; Tschöp et al., 2000; Wren et al., 2001). Consistent with previous studies (Tschöp et al., 2000; Wren et al., 2001), i.p. injection of ghrelin (1 μg/g) increased food intake prominently during the early light period (Figures 1D–1F) and increased feeding slightly during the early dark period (Figure S4C). We thus predicted that astrocyte activation would reduce ghrelin-evoked feeding. As expected, we found that prior administration of CNO robustly reduced the ghrelin-evoked food intake during both light and dark periods (Figures 1E and S4C). We also examined whether astrocyte activation inhibits food intake during post-fasting refeeding. We found that astrocyte activation reduced food intake during the late phase of refeeding, but not during the early phase of refeeding (Figures S5A and S5B). The early voracious food intake immediately post-fasting probably resulted from the integration

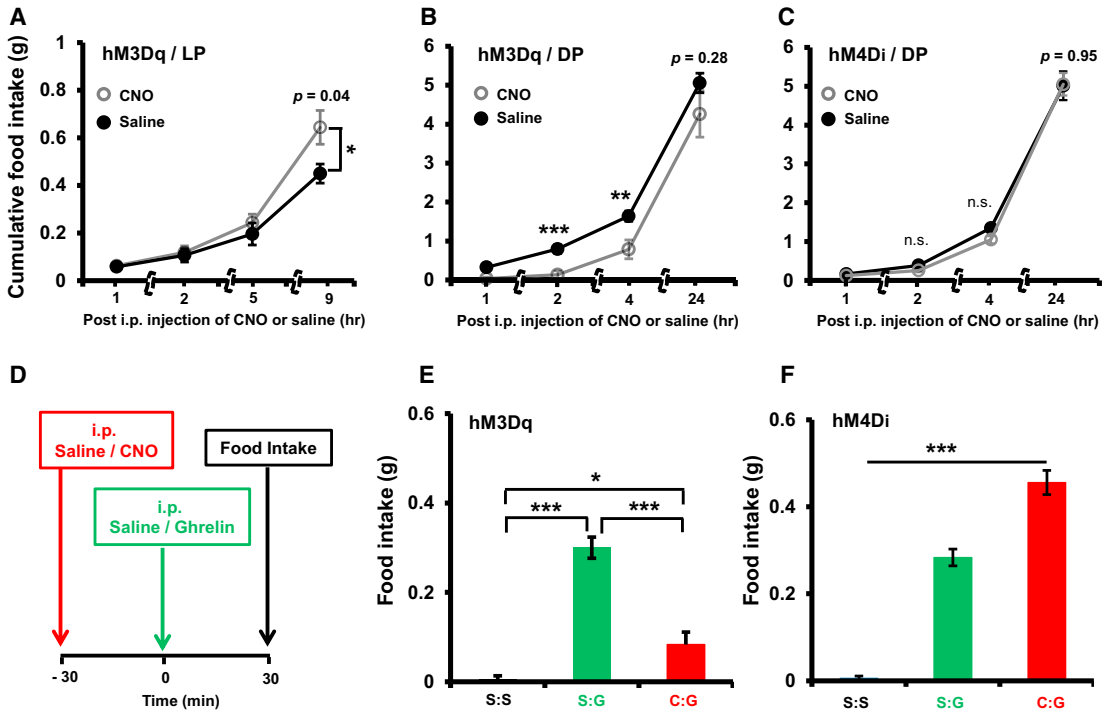

Figure 1. Astrocytic Bi-directional Regulation of Food Consumption

(A and B) The i.p. injection of CNO had no apparent effect on basal food intake (FI) during the early light period (A; n = 8; CNO to saline: unpaired t test, $t_{14} = 0.30$, p = 0.62 for 2-hr FI), while significantly reduced FI occurred at the start of the dark period (B; n = 8; CNO to saline: unpaired t test, $t_{14} = 4.0$, $p < 0.001$ for 2-hr FI; $t_{14} = 3.0$, p = 0.006 for 4-hr FI) in the mice transduced with DREADD-hM3Dq in medial basal hypothalamic astrocytes, compared to mice that received saline injections. There was a rebound of feeding within 9 hr post-CNO injections (A) and no difference in the daily FI (B).

(C) CNO-based astrocyte inactivation did not affect basal FI in the mice transduced with DREADD-hM4Di (n = 6; CNO to saline: p = 0.06 for 2-hr or 4-hr FI, respectively).

(D) Experimental design. The mice first received injection of saline or CNO, respectively, and 30 min later received the second injection of saline or ghrelin, respectively. The 30-min food consumption was measured post-injection of saline or ghrelin.

(E and F) Ghrelin-evoked feeding was significantly depressed by CNO-based astrocyte activation (E; n = 7; $F_{2,18} = 55.34$, $p < 0.001$; S:S to C:G, $F_{1,12} = 7.42$, $p < 0.05$), whereas potentiated by astrocyte inactivation (F; n = 9; $F_{2,24} = 135.72$, $p < 0.001$). Data are represented as mean ± SEM. All the data were collected from the same mice before and after different treatments in each group. LP, light period; DP, dark period; S:S, two i.p. injections of saline at the intervals of 30 min; S:G, saline and ghrelin; C:G, CNO and ghrelin; n.s., not significant (*$p < 0.05$, ***$p < 0.001$).

of multiple pathways of the signaling originating from peripheral tissues and multiple groups of the cells localized both inside and outside of the hypothalamus. The integration of these signaling pathways probably exerts strong appetite to evoke food intake by overcoming astrocytic inhibition of feeding. Also, it is likely that the cellular depletion of ATP after food deprivation diminished the inhibition of food intake via astrocyte regulation.

Astrocyte Inactivation Enhanced and Prolonged Ghrelin-Evoked Feeding

Next, we examined whether and how inactivating astrocytes affects food intake. Specific expression of the engineered muscarinic receptor variant hM4Di in astrocytes was achieved by injecting GFAP-driven viral vectors into the medial basal hypothalamus. The engineered receptor hM4Di couples through the G_i pathway that hyperpolarizes cells (Armbruster et al., 2007; Sweger et al., 2007) by diminishing the constitutive GPCR-mediated intracellular signaling pathways required for the tonic release of gliotransmitters (Agulhon et al., 2012; Shigetomi et al., 2013). We proposed that these signaling pathways within the astrocytes expressing hM4Di would be constitutively inactivated by CNO, and thereafter we used this as a means to inactivate astrocyte. After 2 weeks to allow for hM4Di-mCherry expression, we performed behavioral experiments on the transduced mice. The basal food intake was apparently not affected (Figure 1C). Surprisingly, in contrast to the astrocyte activation-induced reduction of food intake (Figure 1E), astrocyte inactivation prominently enhanced and prolonged ghrelin-evoked feeding (Figure 1F). These experiments were repeated under blinded and unblended conditions with similar results (Figure S5C). Collectively, these findings indicate that the astrocytes within medial basal hypothalamus do in fact participate in regulating feeding behaviors.

Astrocytes Facilitated Leptin-Regulated Feeding Behaviors

In contrast to ghrelin, leptin exerts negative effects on food intake. Recent studies indicate that astrocytes express leptin

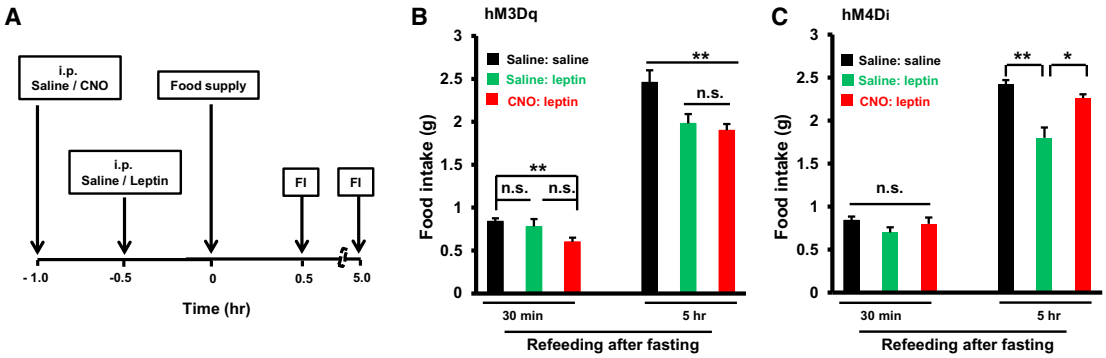

Figure 2. Astrocytic Regulation of Leptin-Induced Feeding Behavior

(A) Experimental design. The i.p. injection of leptin or vehicle was administered 30 min after the first injection of CNO or saline, respectively. The animal food was supplied 30 min after leptin or vehicle injection, and food consumptions were measured at 30 min and 5 hr, respectively, after food supply.

(B) CNO-based astrocyte activation prominently facilitated leptin-induced anorexia, while leptin in its own had no apparent effects on 30-min FI after refeeding post-fasting (n = 5 for each group; leptin to vehicle, $F_{1,8} = 0.42$, p = 0.53; CNO/leptin to vehicle, $F_{1,8} = 15.57$, p = 0.004; leptin to CNO/leptin, $F_{1,8} = 3.36$, p = 0.10). However, leptin significantly reduced 5-hr FI (n = 5 for each group; $F_{2,12} = 7.54$, p = 0.008; leptin to CNO/leptin, $F_{1,8} = 0.37$, p = 0.56).

(C) CNO-based astrocyte inactivation blunted leptin-induced anorexia (n = 5 for each group; unpaired t test for 5-hr FI, saline/vehicle to saline/leptin, $p < 0.01$; CNO/leptin to saline/leptin, $p < 0.05$). *$p < 0.05$, **$p < 0.01$; n.s., not significant.

receptors (*Lepr*) (Kim et al., 2014; Hsuchou et al., 2009) and that leptin-regulated feeding is diminished in mice lacking astrocyte-specific leptin receptors (Kim et al., 2014). It also was reported that leptin activates hypothalamic astrocytes by elevating astrocytic calcium levels (Hsuchou et al., 2009). We suspect therefore that leptin-induced anorexia results, at least in part, in activation of astrocytes via leptin-mediated signaling. We then tested the effects of chemical-genetic activation or inactivation of astrocytes on leptin-regulated feeding behaviors. We found that activating astrocytes facilitated leptin (3 μg/g)-induced inhibition of food intake (Figures 2A and 2B), whereas inactivating astrocytes blunted leptin-induced anorexia (Figures 2A and 2C). These results are opposite those of astrocyte activation or inactivation on ghrelin-evoked hyperphagia, respectively (Figure 1). Our data together with the previous report (Kim et al., 2014) suggest that astrocytes play an important role in energy homeostasis by potentiating anorexigenic leptin-induced anorexia and depressing orexigenic ghrelin-induced hyperphagia, respectively.

Blood-Brain Barrier Did Not Participate in Astrocytic Inhibition of Feeding

Next, we probed the underlying mechanism(s) of astrocyte activation-induced inhibition of food intake. We first considered the possibility that astrocyte activation-induced anorexia might be caused by a decreased permeability for ghrelin across the blood-brain barrier (BBB). To test this, we used intra-ARC injection technique to directly administrate ghrelin into the ARC or medial basal hypothalamus through a bilateral cannula (Figures S5D and S5E) on the mice transduced with hM3Dq or hM4Di in the medial basal hypothalamic astrocytes. We found that intra-ARC injections of ghrelin evoked as much food intake (Figures 3A–3C) as that observed in response to i.p. injections of ghrelin (Figures 1D–1F). In addition, consistent with i.p. injection of ghrelin (Figures 1D–1F), the food intake by intra-ARC ghrelin was reduced by astrocyte activation, whereas it was increased by astrocyte inactivation (Figures 3B and 3C).

Additional work will be needed to further understand the mechanism(s) of astrocyte inactivation-based enhancement in ghrelin-evoked feeding. Thus far, our results demonstrate that (1) the medial basal hypothalamus is one of the brain regions through which ghrelin evokes food intake, consistent with the notion that AgRP neurons are required for ghrelin-evoked feeding (Luquet et al., 2007); (2) astrocyte activation or inactivation depresses or facilitates ghrelin-evoked feeding, respectively; and (3) the BBB is not involved in astrocytic regulation of feeding. We thus assume that astrocytes regulate feeding behaviors probably by modulating the extracellular levels of gliotransmitter(s) in medial basal hypothalamus.

Adenosine A_1 Receptor Mediated Astrocyte Activation-Induced Anorexia

We next sought to determine which gliotransmitter(s) is responsible for astrocytic-dependent inhibition of feeding. We first considered adenosine since it negatively regulates synapse transmissions and inhibits neuronal activity by activating adenosine A_1 receptors located on pre- and post-synaptic neurons (Chen and van den Pol, 1997; Liu and Gao, 2007; Obrietan et al., 1995; Thompson et al., 1992). To test if endogenous adenosine exerts a tonic inhibition on food intake, we used DPCPX, a selective adenosine A_1 receptor antagonist, to block the endogenous extracellular adenosine (Bekar et al., 2008). We found that intra-ARC injections of DPCPX profoundly enhanced and prolonged ghrelin-evoked feeding, which lasted several hours (Figures 4A and 4B). DPCPX just transiently increased basal food intake within 30 min post-administration (Figure 4C). As expected, DPCPX also increased the food intake during the refeeding post-fasting (Figures S6A and S6B). Moreover, DPCPX diminished the inhibition of astrocytes on food intake several hours later after the refeeding compared with the vehicle control group (Figures S6A and S6B).

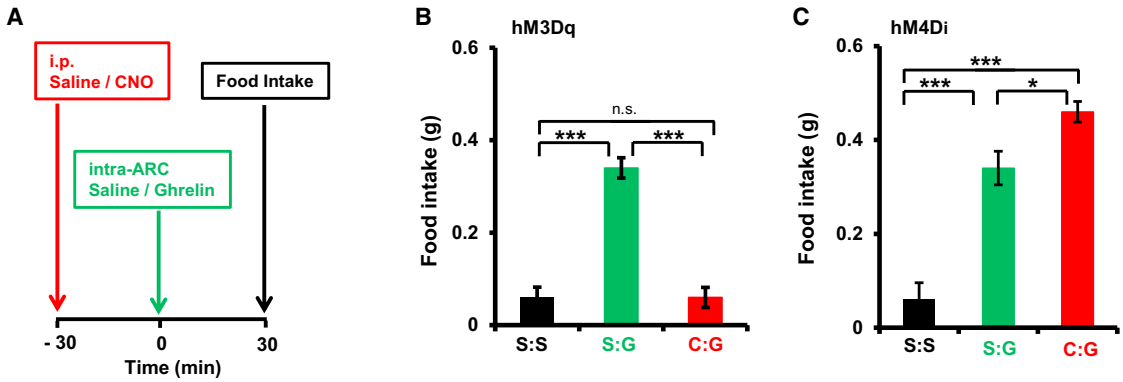

Figure 3. BBB Did Not Contribute to Astrocytic Inhibition of Ghrelin-Evoked Feeding

(A) Experimental design. Intra-ARC injection of saline or ghrelin was used to directly deliver saline or ghrelin to the ARC. Food consumption was measured after the intra-ARC injection of saline or ghrelin.

(B) CNO-based astrocyte activation reduced the intra-ARC ghrelin-evoked feeding in the mice transduced with hM3Dq in the astrocytes, in comparison to that from saline injections (n = 5; $F_{2,14} = 52.27$, $p < 0.001$).

(C) Astrocyte inactivation potentiated the ghrelin-evoked feeding in the mice encoded with hM4Di (n = 5; $F_{2,14} = 41.31$, $p < 0.001$). *$p < 0.05$, ***$p < 0.001$.

Collectively, these findings indicate that the endogenous adenosine downregulates basal and ghrelin-evoked food intake. It is likely that DPCPX-based prolongation of ghrelin-evoked feeding probably resulted from its antagonizing adenosine-mediated inhibition of the feeding. Next, we tested if endogenous adenosine was implicated in the astrocytic inhibition of ghrelin-evoked feeding behaviors. As expected, we found that intra-ARC injections of DPCPX strongly diminished astrocyte activation-based reduction of ghrelin-evoked feeding (Figures 5A and 5B), suggesting that astrocyte counter-regulates ghrelin-evoked feeding by elevating extracellular levels of adenosine derived from the released ATP within milliseconds (Dunwiddie et al., 1997). Cellular depletion of ATP after food deprivation supports our observation (Figures S5A and S5B) that astrocyte activation failed to inhibit the food intake during the early phase of refeeding post-fasting, but did inhibit the feeding several hours later after refeeding during which time the cellular ATP levels recover (Ji and Friedman, 1999).

Astrocyte Activation Inactivated AGRP Neurons via Adenosine A_1 Receptor Signaling

It is well documented that astrocytes actively modulate neuronal synaptic inputs in the CNS (Clarke and Barres, 2013; Horvath et al., 2010; Fields and Stevens-Graham, 2002; Pascual et al., 2005; Stellwagen and Malenka, 2006; Zonta et al., 2003). However, it is not known whether and how astrocytes influence the firing rate of AGRP neurons. To study the cellular mechanism(s) underlying the rapid astrocytic inhibition of food intake, we used the chemical-genetic DREADD approach in which astrocytes were expressed with hM3Dq or a control fluorescent protein mCherry. Using an in vitro protocol, we performed loose-patch clamp recordings on the AGRP neurons in the acute brain slices of NPY-hrGFP transgenic mice.

We observed that transient exposure of the brain slices to CNO (10 μM) led to a prominent reduction in the action potential firing rate of AGRP neurons that lasted several minutes (Figures 6A and 6B). Pre-incubation of the brain slices with DPCPX (1 μM) blocked the inhibition of the firing rate of AGRP neurons by CNO-based astrocyte activation (Figures 6C and 6D), indicating that adenosine A_1 receptors mediated astrocytic inactivation of AGRP neurons, consistent with the previous report that adenosine exerted approximately 90% inhibition of synapse transmissions and neuron firing rate (Thompson et al., 1992). Also, recent studies indicated that selective glial stimulation can exert long-term depression of synapse transmission (Chen et al., 2013; Sasaki et al., 2012). Although the underlying mechanisms are different, these results support our data that CNO activation of astrocytes inhibited AGRP neuron activity for minutes (Figures 6A and 6B). The influence of glial stimulation on neuronal activity may depend on the intensity of stimulation. As expected, we found that the firing rate of AGRP neurons was partially and transiently reduced using CNO at a dose of 2 μM (Figure S6C). CNO did not exert apparent effects on the firing rate of AGRP neurons from the mice in which the astrocytes were encoded with the control fluorescent protein mCherry (Figure 6E). Together, these data indicate that astrocyte activation inactivated AGRP neurons through adenosine A_1 receptor signaling in a dose-dependent manner.

DISCUSSION

We took advantage of a chemical-genetic DREADD approach that allowed us to temporally and spatially activate or inactivate the medial basal hypothalamic astrocytes. The cell-type specificity was demonstrated by showing that hM3Dq-mCherry or hM4Di-mCherry was expressed in only GFAP-positive cells, but not in NSE-expressing neurons (Figure S1). Importantly, there was no expression of hM3Dq or hM4Di in either AGRP or POMC neurons (Figure S2). In the CNS, GFAP expression is commonly used to identify differentiated multipolar astrocytes in vivo and in vitro. Although we cannot exclude the possibility of progenitor cells expressing GFAP in the CNS (Garcia et al., 2004), it has been well demonstrated that astrocytes exert rapid modulation of neuronal activity, whereas progenitor cells do not.

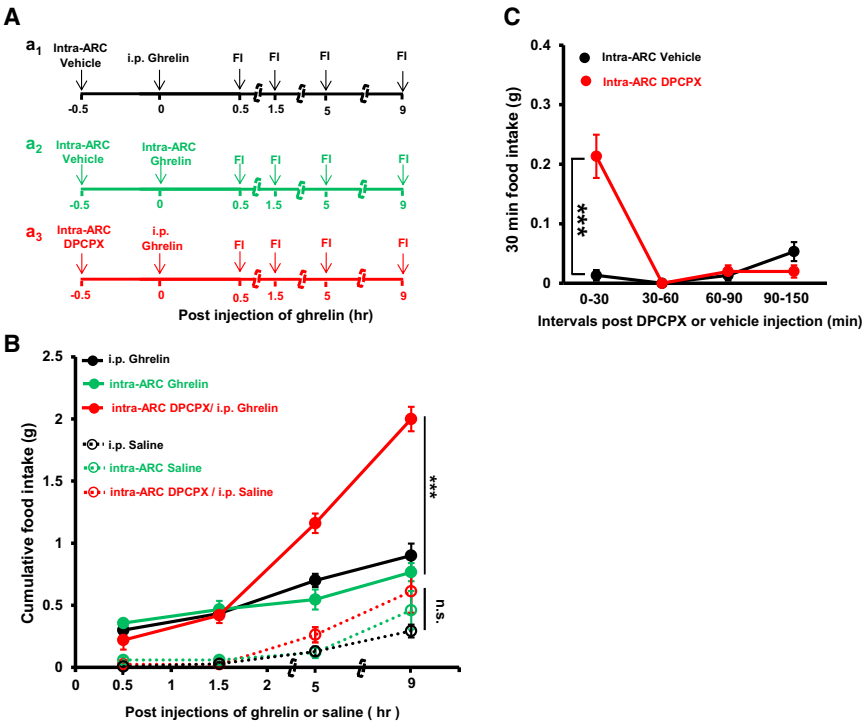

Figure 4. Endogenous Adenosine Counter-Regulated Ghrelin-Evoked Feeding via A_1 Receptors

(A) Experimental design. Cumulative FI was measured at different time points post-ghrelin administration by i.p. (a_1 and a_3) or intra-ARC (a_2) injection. Intra-ARC injection of vehicle (a_1 and a_2) or DPCPX (a_3) was applied 30 min prior to ghrelin injections.

(B) Pre-administration of a vehicle control, intra-ARC ghrelin injection (green) evoked as much FI as that by i.p. injection of ghrelin (black), while pre-administration of DPCPX significantly enhanced and prolonged ghrelin-evoked FI, in comparison to that with the control vehicle pre-treatment (n = 6 for each group; intra-ARC vehicle:i.p. ghrelin, 0.90 ± 0.10; intra-ARC vehicle:intra-ARC ghrelin, 0.80 ± 0.07; intra-ARC DPCPX:i.p. ghrelin, 2.0 ± 0.10; $F_{2,17}$ = 55.69, p < 0.001 for the 9-hr FI). DPCPX pre-treatment did not show significant increase in the 2-hr FI after ghrelin injection. DPCPX-based facilitation on ghrelin-evoked FI happened several hours later after ghrelin treatment. Control experiments were conducted with the injections of saline instead of ghrelin. There was no significant difference in the FI (n = 6), suggesting that DPCPX in itself did not prolong the feeding.

(C) Food consumptions were measured at different time points post-intra-ARC injections of DPCPX or vehicle. DPCPX transiently increased FI within 30 min post-injection (n = 15; vehicle to DPCPX: unpaired t test, t_{28} = 5.17, p < 0.001 for 30-min FI post-injections). All the data were collected from the same mice with different treatments in each group. ***p < 0.001; n.s., not significant. Data are represented as mean ± SEM.

We therefore conclude that astrocytes comprise the vast majority of the cells implicated in the regulation of feeding behaviors and of AGRP neuron activity in our experiments. The GFAP promoter has been used before to express proteins in astrocytes. The astrocytic specificity of the GFAP promoter was questioned in a recent study (Fujita et al., 2014). However, because of potential compensation mechanisms that occur in knockin and knockout transgenic mice, we are concerned that the mutant mice may not accurately reflect the situation in the wild-type, and thus it remains controversial. For example, cell-type specificity of GFAP-positive cells may be influenced during the developmental switch of targeted cells. Thus, the cell-type specificity of GFAP-driven expression in transgenic mice may differ from that of the virally transduced adult mice that we used in our studies.

Regulation of feeding behaviors is crucial for energy homeostasis. In this report, we provide the evidence demonstrating that the medial basal hypothalamic astrocytes control feeding behavior bi-directionally, regulating appetite under both favorable and unfavorable conditions. For instance, our data showed that the orexigenic ghrelin-induced hyperphagia was depressed by activating astrocytes but potentiated by inactivating astrocytes (Figures 1, 3, and 5), while the anorexigenic leptin-induced anorexia was facilitated by astrocyte activation but blunted by astrocyte inactivation (Figure 2). Interestingly, we also observed that astrocyte activation did not affect the voracious food intake during the early phase of refeeding post-fasting, but reduced the feeding several hours after the onset of refeeding (Figures S5A and S5B). These results suggest that (1) fasting-induced starvation may lead to the changes of both hormonal and nutrient signals originating in peripheral tissues, and (2) fasting also may change the activities of vagal afferent neurons and other types of cells responsible for appetite. These changes probably directly affect the appetite control circuits both inside and outside of the hypothalamus to evoke voracious food intake with overcoming the astrocytic inhibition of the hunger circuits within medial basal hypothalamus.

We also considered the temporal recovery of cellular ATP during refeeding post-fasting, since ATP-derived adenosine reduces synaptic strength and the firing rate of neurons (Chen and van den Pol, 1997; Liu and Gao, 2007; Obrietan et al., 1995; Thompson et al., 1992). The neuronal electrical activities are crucial for orchestrating feeding (Aponte et al., 2011; Atasoy et al., 2012; Kong et al., 2012; Schneeberger et al., 2013). Although unknown in the CNS, in the liver it takes approximately 6 hr after the onset of refeeding for ATP levels to recover (Ji and Friedman, 1999), which is temporally consistent with the time-dependent astrocytic inhibition of the food intake we observed after refeeding (Figure S5B). This is further demonstrated by our pharmacological experiments that adenosine mediates astrocytic inhibition of food intake (Figures 4 and 5). For instance, we found that the endogenous adenosine exerted a tonic inhibition on basal food intake and counter-regulated the ghrelin-evoked feeding by acting on the A_1 receptors to inactivate the orexigenic AGRP neurons in the ARC (Figures 4, 5, and 6).

Adenosine also may exert modulatory effects on the activities of other types of neurons, such as POMC neurons, which

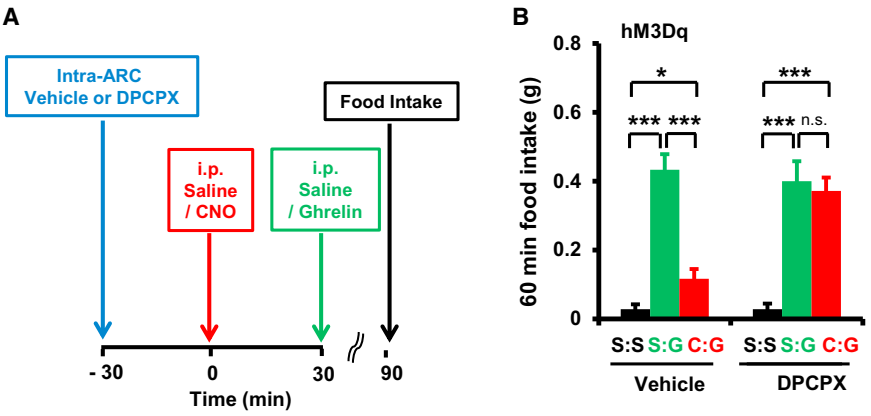

Figure 5. Adenosine A_1 Receptors Mediated Astrocytic Inhibition of Ghrelin-Evoked Feeding

(A) Experimental design. The mice received three injections daily, respectively, through intra-ARC or i.p. administration of vehicle or DPCPX (first injection, blue), saline or CNO (second injection, red), and saline or ghrelin (third injection, green). One-hour FI was measured after the third injection of saline or ghrelin.

(B) In the presence of the vehicle, CNO stimulation of astrocytes depressed ghrelin-evoked feeding ($n = 6$; $F_{2,17} = 43.48$, $p < 0.001$). In contrast, in the presence of DPCPX, astrocytic inhibition of ghrelin-evoked feeding was significantly abolished ($n = 7$; $F_{2,20} = 25.13$, $p < 0.001$). *$p < 0.05$, ***$p < 0.001$; n.s., not significant. Arrows in (A) indicate the time points of injections or food measurement.

negatively regulate feeding behaviors (Aponte et al., 2011). Because of the rapid inhibition of astrocyte activation on feeding, we focused on the effects of astrocytes on the firing rate of AGRP neurons, which rapidly orchestrate feeding behaviors. Control experiments excluded the non-specific influence on food intake possibly brought on by CNO administration or intra-brain injections. We did not observe changes in the food intake of the mice transduced with the control fluorescent protein mCherry (Figures S4, S5A, S5B, S6A, and S6B). Also, there were no significant changes in the basal or ghrelin-evoked food intake between the non-injected and injected mice or mice inserted with the guide cannula (data not shown).

Based on these experiments, we propose a model in which astrocytes, responding to either hormonal or neuronal signals evoking food intake, release gliotransmitter(s) to modulate synaptic strength and the firing rate of AGRP neurons (Figures 6F–6H). For instance, the orexigenic ghrelin evokes feeding by increasing the activities of AGRP neurons through facilitating presynaptic release of glutamate (Abizaid et al., 2006; Yang et al., 2011). The increased glutamate inside the synaptic cleft may spill over to activate the glutamate receptors localized on the astrocytes surrounding the synapses, such as the ionotropic and metabotropic glutamate receptors (Biber et al., 1999; D'Ascenzo et al., 2007; Seifert and Steinhäuser, 2001). Activation of the glutamate receptors may trigger gliotransmitter release, such as ATP (Bal-Price et al., 2002). The released ATP is degraded rapidly (within ~200 ms) into adenosine by a series of ectonucleotidase (Dunwiddie et al., 1997).

Although we cannot exclude the transient effects of ATP on neuronal activities and feeding, we assumed that astrocyte activation negatively regulates ghrelin-evoked feeding by increasing extracellular adenosine to inactivate AGRP neurons. This idea also is supported by a previous report that shows adenosine inhibits GABA release in solitary self-innervating arcuate neurons (Chen and van den Pol, 1997). The inhibitory neurotransmitter GABA is co-expressed with neuropeptide Y (NPY) in the NPY/AgRP neurons in the ARC (Horvath et al., 1997). In contrast, loss of A_1 receptor functions by genetic or pharmacological means may facilitate ghrelin-evoked feeding due to lack of adenosine-mediated inhibition of orexigenic neurons (Figure 6H). Our data also may explain why a single injection of ghrelin only transiently increases prominent feeding within 30 min post-injection (Tschöp et al., 2000; Wren et al., 2001). Our model suggests that astrocytes may constitute a valuable target for the development of therapeutic interventions to ghrelin resistance (Luquet et al., 2007).

We proposed that the medial basal hypothalamic astrocytes serve as a checkpoint of energy states by bi-directional regulation of feeding behaviors, thus preventing from energy deficit or surfeit. This report fills an important gap in our understanding of the functional roles of glial cells in regulating food intake, and it provides insights into the dynamics of feeding. Further investigation into the astrocytic scaling modules described here and the use of astrocyte-specific manipulation techniques may be useful toward controlling obesity and other eating disorders, such as anorexia.

EXPERIMENTAL PROCEDURES

All the experimental procedures were approved by the Institutional Animal Care and Use Committee of State University of New York Upstate Medical University, according to U.S. NIH guidelines for animal research. For the experiments performed in Figure S5C, the experimenter was blinded to the identity of the pharmacological reagent.

Animals

All NPY-hrGFP, POMC-EGFP, and C57BL/6J mice were purchased from The Jackson Laboratory. The transgenic mice were genotyped for Gfp. Only male mice were used. All the mice were kept in temperature- and humidity-controlled rooms on a 12-hr:12-hr light-dark cycle, with lights on from 7:00 a.m. to 7:00 p.m. Mouse chow (PicoLab Rodent Diet 20, 5053 tablet, TestDiet) and water were provided ad libitum unless otherwise noted. The mice were single-caged once they received viral injections or were inserted with the guide cannula until all the experiments on the mice were finished.

Pharmacology

All the chemicals were purchased from Sigma except that ghrelin (human) was purchased from Bachem, leptin was from Tocris, and CNO was purchased from Enzo Life Sciences. For the experiments requiring intra-ARC injections, a bilateral injector (33 GA, Plastics One) with 1-mm extension beyond the guide cannula (4.7 mm, 26 GA, Plastics One) was attached by polyethylene tubing to

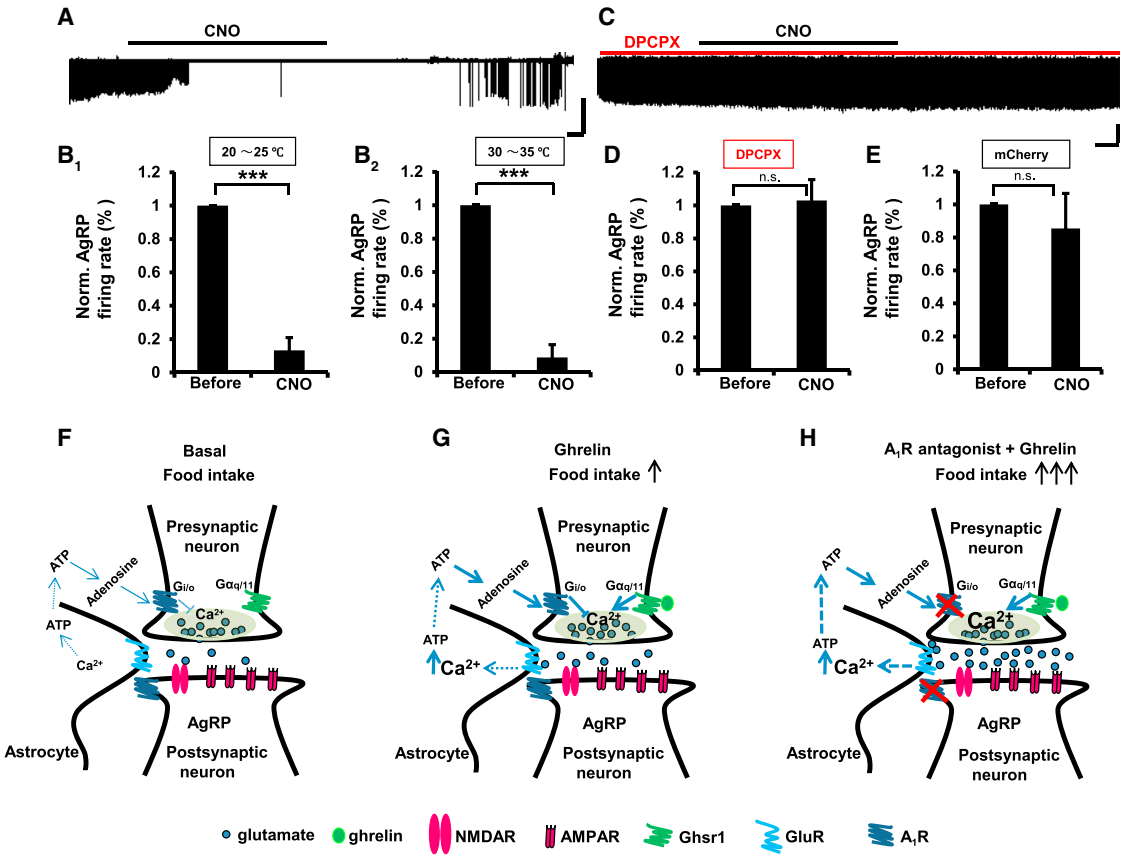

Figure 6. Astrocyte Activation Inactivated AGRP Neurons via A_1 Receptors

(A) A sample trace shows that addition of CNO (10 μM) to the acute brain slices from the mice transduced with DREADD-hM3Dq in the medial basal hypothalamic astrocytes significantly reduced the firing rate of AGRP neuron.

(B) The temperature did not show apparent effects on the astrocytic reduction of the firing rate, as indicated by the grouped data at room temperature (left; n = 5; $F_{1,9}$ = 130.95, p < 0.001) and physiological temperature (right; n = 4; $F_{1,7}$ = 147.87, p < 0.001).

(C and D) A sample electrophysiological recording trace (C) shows that pre-incubation of the brain slice with DPCPX diminished CNO-based inhibition of AgRP neuron firing rate, as also indicated by the grouped data (D; n = 6; $F_{1,11}$ = 0.05, p = 0.82).

(E) CNO addition did not exert significant effects on AGRP neuron firing rate in the mice transduced with a control fluorescent protein mCherry in the astrocytes (n = 4; $F_{1,7}$ = 0.49, p = 0.51).

(F–H) A proposed model of glial relay circuit counter-regulating ghrelin-evoked feeding. (F) Neuron-astrocyte signaling under basal FI. (G) While ghrelin evokes FI by evoking glutamate release from presynaptic terminals, neighboring astrocytes respond to glutamate via glutamate receptors localized on astrocytes with elevating Ca^{2+} signaling to trigger gliotransmitter release, such as ATP. The released ATP is converted to adenosine within milliseconds to inactivate AgRP neurons by acting on A_1 receptors localized on pre- and post-synaptic neurons. (H) Loss of A_1 receptor functions can enhance and prolong ghrelin-evoked feeding. ***p < 0.001. Scale bar, 250 pA and 1 min.

a Hamilton syringe. For all the bilateral intra-ARC injections, 200 nl (each side) vehicle or chemicals were finished within 5 min for each mouse. For the experiments requiring i.p. injections, we used 27-gauge needles and the stocks of the chemicals (ghrelin, leptin, or CNO) were diluted in saline, respectively, on the experimental days. Injections into the abdomen were made at a 30° angle, and the shaft of the needle entered to a depth of about 5 mm.

Stereotaxic viral delivery, feeding assays, electrophysiology, and imaging methods are provided in the Supplemental Experimental Procedures.

Statistical Analysis

Data are represented as mean ± SEM and error bars also indicate SEM. Statistical analyses were performed by use of Prism 6.0 software (GraphPad). P values were calculated by unpaired two-tailed Student's t test or one-way or two-way ANOVA. For detection of the firing rate of AGRP neurons, we used template matching (Clampfit, Molecular Devices) followed by visual inspection (*p < 0.05, **p < 0.01, ***p < 0.001; n.s., not significant).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.04.002.

AUTHOR CONTRIBUTIONS

Y.Y. conceived the study idea. L.Y. and Y.Y. designed the study. L.Y., Y.Q., and Y.Y. performed the experiments, analyzed the data, and wrote the paper.

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